Exhibit 4

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Simultaneous presence of tissue factor pathway inhibitor (TFPI) and low molecular weight heparin has a synergistic effect in different coagulation assays

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Injection of heparin releases tissue factor pathway inhibitor (TFPI) to the blood and, after heparin neutralization, it has been recently demonstrated that the released TFPI has an anticoagulant activity. Using recombinant TFPI (rTFPI) we have investigated how the simultaneous presence of TFPI and low molecular weight heparin (LMW heparin) affects different coagulation assays. Coagulation was measured using the activated partial thromboplastin time, the prothrombin time and a dilute tissue factor assay. The anticoagulant activity of partly purified plasma TFPI (pTFPI) was much higher than that of TFPI. However, this high anticoagulant activity was unstable, so in order to investigate the effect of pTFPI and LMW heparin we used an inhibitory antibody towards TFPI and looked at the effect of removing TFPI from plasma. When both rTFPI and LMW heparin was added to plasma a synergistic effect was observed in all assays. In the tissue factor dependent coagulation assays, the effect of adding rTFPI or removing pTFPI was more pronounced in the presence of heparin. TFPI plays a significant role in assays where the coagulation time is prolonged for some reason. This may be caused by dilution of tissue factor, by the presence of heparin or by a defect in the coagulation cascade such as that seen in haemophilia.

Key words: Tissue factor pathway inhibitor, low molecular weight heparin, coagulation assays, coagulation inhibition.

Introduction

Tissue factor pathway inhibitor (TFPI) previously extrinsic pathway inhibitor (EPI) or lipoprotein associated coagulation inhibitor (LACI) plays an essential regulatory role in initiation of the extrinsic coagulation pathway by inhibiting the proteolytic activity of factor VIIa/tissue factor and factor Xa.[1] TFPI is a 43 kDa glycoprotein that contains three tandem Kunitz type inhibitory domains.[2] The majority of TFPI normally circulates in plasma in complex with lipoproteins,[3] and the plasma concentration of TFPI is approximately 0.05 μg/ml.[4]

Heparin is a polysaccharide chain composed of repeating sulphated glucosamine glucuronic acid or iduronic acid in disaccharide sequences. Heparin acts as an anticoagulant by forming complexes with antithrombin III and heparin cofactor II and thereby potentiates the inhibition of FXa and thrombin. It is debatable whether inhibition of thrombin or FXa is most important for the anticoagulant and antithrombotic activity of heparin.[5–8] Recently it was demonstrated that injection of heparin releases TFPI into the blood.[9] After neutralizing the heparin with polybrene, the released TFPI itself has an anticoagulant activity.[10] Broze *et al.*[11] have shown that binding of TFPI to human FXa is increased upon addition of heparin. We have now investigated how the simultaneous presence

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of low molecular weight heparin (LMW heparin) and TFPI affects different coagulation assays. For these studies recombinant TFPI (rTFPI) and an inhibitory antibody towards TFPI were used.

Materials and methods

Reagents

APTT IL test was purchased from Instrumentation Laboratory, Ascoli Piceno, Italy. Rabbit brain thromboplastin and bovine factor X were from Sigma Diagnostics, St Louis, MO, USA. LMW heparin (Logiparin®), porcine mucosal sodium heparin, and recombinant factor VIIa were from Novo-Nordisk A/S, Gentofte, Denmark. Sepharose 4B was purchased from Pharmacia LKB, Uppsala, Sweden and S-2222 from Kabi Vitrum, Stockholm, Sweden. Human brain thromboplastin was prepared from human brain acetone powder[12] and was kindly provided by Dr Peter Wildgoose (Novo-Nordisk A/S). Normal pooled human plasma was obtained from healthy donors and was stored at −80°C.

Recombinant TFPI was obtained from the culture medium of transfected BHK cells and was purified by a combination of heparin–Sepharose chromatography, ion-exchange chromatography and reverse phase HPLC.[4] A 2 μg/ml solution of our BHK-derived rTFPI prolonged the clotting time of normal human plasma from 35 s to 65 s when measured in a dilute tissue factor assay using rabbit thromboplastin. A similar activity has been reported for rTFPI obtained from C 127 cells.[13]

Polyclonal anti-TFPI antibody was obtained by immunization of rabbits with rTFPI, and the IgG fraction isolated by caprylic acid precipitation.[10] The specificity of this antibody has been carefully examined to ensure that it specifically inhibits TFPI activity as measured in a chromogenic TFPI activity assay and in coagulation assays.[14]

Preparation of plasma TFPI

Plasma TFPI was partly purified from normal pooled human plasma using affinity chromatography. In this procedure 1.4 l of plasma was passed through a 20 ml column of anti-TFPI IgG Sepharose (4 mg IgG/ml) at a flow rate of 48 ml/h. Following a 250 ml wash with TBS (50 mM Tris, 100 mM NaCl, pH 7.4), bound proteins were eluted with Gly-HCl (100 mM glycine, pH 2.5) and 3 ml fractions collected into 0.45 ml of Tris-buffer (1 M Tris, pH 8). The fractions were analysed in the chromogenic TFPI activity assay, and active fractions pooled. The yield of plasma TFPI using this affinity chromatography method was approximately 60%. The preparation contained 56 units of TFPI/ml, and the specific activity was 111 units of TFPI/mg protein. Thus the purification factor from plasma was 8000-fold.

Chromogenic TFPI activity assay

TFPI was measured by a two step chromogenic assay[15] adapted for microplates.[4] In the first step, the samples were incubated with 100 μl of FVIIa/tissue factor/FX/$CaCl_2$ combination reagent at room temperature for 10 min. In the second step an excess of FX (40 nM) was added, and the FXa generation measured by using a chromogenic substrate (S-2222). As a standard we used a pool of normal plasma assigned a value of 1 U/ml of TFPI. All plasma samples were heat treated for 15 min at 56°C to avoid interference in the assay with other plasma coagulation factors.

Coagulation assays

Coagulation assays were performed on plasma samples with added TFPI, LMW heparin and/or antibody, all diluted in coagulation buffer (0.1% bovine serum albumin, 50 mM imidazole, 100 mM NaCl, pH 7.3). One-sixth of the total volume was rTFPI, 1/20 of the volume was LMW heparin and 1/20 of the volume was anti-TFPI IgG (1.4 mg/ml). In samples where some of these reagents were not added, coagulation buffer was added to keep the plasma dilution constant. All samples were incubated for 15 min at room temperature before starting the assay. All clotting times were measured on an ACL 300 R coagulation analyser from Instrumentation Laboratories, Ascoli Piceno, Italy.

All the experimental curves (Figures 1–3, 5, 6) obtained by coagulation assays were the results of single experiments. All experiments were repeated three to five times with similar results to ensure that the conclusions were not based on isolated observations.

APTT assay. In the activated partial thromboplastin time (APTT) assay, 55 μl of plasma incubation mixture was mixed with 55 μl of APTT reagent for 300 s at 37°C before adding 55 μl of 0.025 M $CaCl_2$ and measuring the coagulation time.

PT assay. In the prothrombin time (PT) assay, rabbit thromboplastin was dissolved according to the manufacturers' instructions and 1 vol of thromboplastin was mixed with 2 vol of 0.03 M $CaCl_2$. In the assay, 75 μl of incubation mixture was mixed with 75 μl of thromboplastin/$CaCl_2$ reagent at 37°C before the clotting time was measured.

Dilute tissue factor (dTF) assay. The dTF assay was similar to the PT assay, except that human thromboplastin diluted 7000-fold in coagulation buffer replaced the undiluted rabbit thromboplastin used in the PT.

Results

APTT assay

TFPI is a direct inhibitor of FXa. Therefore the effect of adding rTFPI together with heparin was investigated even though the APTT is considered to be an assay for the intrinsic coagulation system. Figure 1 shows the effect of adding increasing concentrations of rTFPI and LMW heparin, alone or in combination, to normal human plasma. Addition of rTFPI (10 µg/ml) or LMW heparin (0.4 FXaI U/ml) increased the APTT of normal human plasma by 26 and 57 s, respectively. Co-incubation of an equal amount of LMW heparin and rTFPI resulted in a much greater effect, prolonging the coagulation time by 283 s. At increasing LMW heparin concentrations, the slopes of the TFPI dose-response curves increased, indicating a synergistic effect between rTFPI and LMW heparin. An increase in slope was also seen if the data from Figure 1 were depicted in a semilogarithmic plot (not shown). However, in the semilogarithmic plot the curves were far from linear and deflected at high TFPI concentrations.

PT assay

The PT was only affected by high concentrations of heparin or rTFPI. Figure 2 shows the effect of adding different concentrations of rTFPI and LMW heparin in the PT assay. Addition of 4 µg/ml of rTFPI to normal plasma prolonged the coagulation time by 4.4 s to 16.6 s. Addition of 2 FXaI U/ml of LMW heparin prolonged the coagulation time by 10.4 s to 23 s. However, combination of rTFPI and LMW heparin prolonged the coagulation time by as much as 141 s to 153 s. Figure 2A shows the PTs depicted in a linear plot. Transferred to a semilogarithmic plot the same data give straight lines (Figure 2B). From both graphs a synergistic effect between rTFPI and LMW heparin can be seen; e.g. 1 FXaI U/ml of LMW heparin pro-

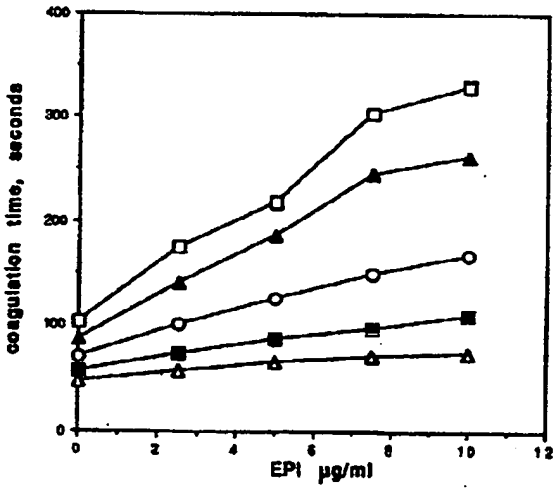

Figure 1. rTFPI dose-response curves at different concentrations of LMW heparin measured in APTT assay. △, 0 FXaI U/ml heparin; ■, 0.1 FXaI U/ml heparin; ○, 0.2 FXaI U/ml heparin; ▲, 0.3 FXaI U/ml heparin; □, 0.4 FXaI U/ml heparin.

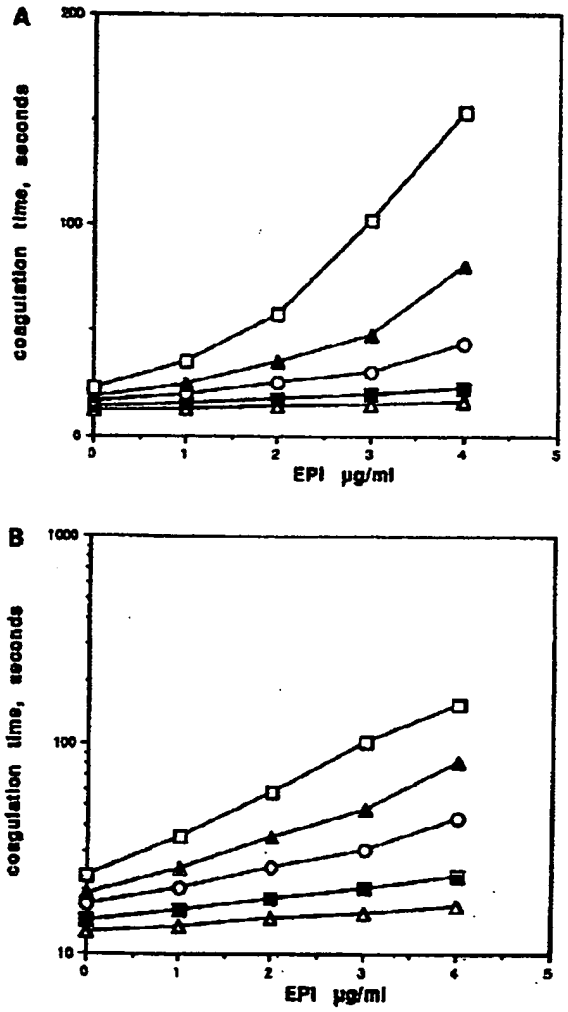

Figure 2. rTFPI dose-response curves at different concentrations of LMW heparin measured in PT assay. (A) Depicted as a lin–lin plot. (B) Depicted as a lin–log plot. △, 0 FXaI U/ml heparin; ■, 0.5 FXaI U/ml heparin; ○, 1.0 FXaI U/ml heparin; ▲, 1.5 FXaI U/ml heparin; □, 2.0 FXaI U/ml heparin.

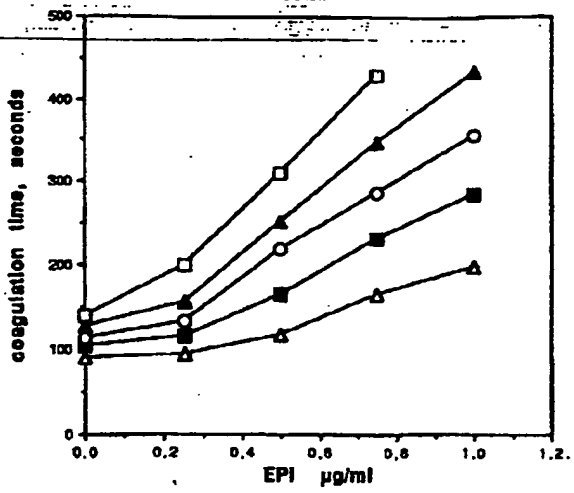

Figure 3. rTFPI dose response curves at different concentrations of LMW heparin measured in dTF assay. △, 0 FXaI U/ml heparin; ■, 0.05 FXaI U/ml heparin; ○, 0.10 FXaI U/ml heparin; ▲, 0.15 FXaI U/ml heparin; □, 0.20 FXaI U/ml heparin.

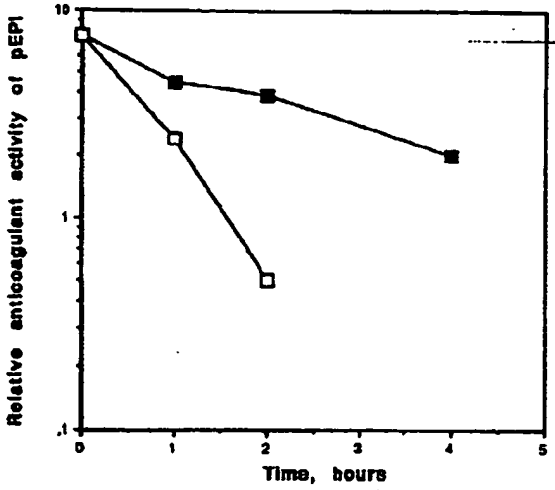

Figure 4. Anticoagulant activity of pTFPI after storing. A preparation of pTFPI in Tris-buffer was split in two and placed at either room temperature or at 4°C. At different times a sample was taken and the anticoagulant activity was measured in the dTF assay and compared with a preparation of rTFPI kept at −20°C. The figure shows the ratio of anticoagulant activity between pTFPI and rTFPI. ■, 4°C; □, 20°C.

longed the PT by 5 s. Double this amount of heparin prolonged the clotting time by 11 s. However, if rTFPI prolonging the clotting time by 5 s (4 µg/ml) was added to plasma containing 1 U/ml of LMW heparin, the clotting time was prolonged by 68 s.

Dilute tissue factor (dTF) assay

In the dTF coagulation assay, coagulation was initiated by tissue thromboplastin as in the PT. Since only trace amounts of thromboplastin were used it resulted in much longer coagulation times than obtained in the PT assay. Furthermore, the thromboplastin used in the dTF assay was of human origin as opposed to the rabbit thromboplastin used in the PT. The coagulation times obtained in the dTF assay are dependent on coagulation factors VII, VIII and IX,[14] and the assay is affected by physiological concentrations of TFPI.

The effect of adding increasing concentrations of rTFPI and LMW heparin, alone or in combination, in the dTF assay is shown in Figure 3. Addition of 1.0 µg/ml of rTFPI to normal plasma prolonged the coagulation time by 105 s to 196 s. Addition of 0.15 FXaI U/ml of LMW heparin to normal human plasma prolonged the coagulation time by 35 s to 126 s. Simultaneous addition of the two, resulted in a prolongation of 341 s to 432 s, and thus also in the dTF assay we obtained results that indicated a synergistic effect between rTFPI and LMW heparin.

Plasma TFPI in dTF coagulation assay

We have previously shown that TFPI partly purified from plasma (pTFPI) has a 15-fold higher anticoagulant activity than rTFPI.[14] For this reason we wanted to look at the effect of of simultaneous addition of pTFPI and heparin in the dTF assay which is relatively sensitive to TFPI. However, the high anticoagulant activity of partly purified pTFPI is unstable. From Figure 4 it can be seen that the half-life of partly purified pTFPI measured in the dTF assay was less than 1 h at room temperature and 2 h at 4°C. Unlike the anticoagulant activity, the chromogenic activity of pTFPI remained stable even after 48 h at room temperature. Because of the stability problems with pTFPI we were not able to perform experiments with simultaneous addition of pTFPI and heparin in coagulation assays. Instead of adding pTFPI we looked at the effect of removing TFPI from plasma by adding anti-TFPI IgG (Figure 5). Addition of 0.4 FXaI U/ml of LMW heparin to normal human plasma prolonged the coagulation time from 97 to 388 s. Simultaneous addition of anti-TFPI IgG to the heparinized plasma shortened the coagulation time to 187 s, a reduction of 201 s just by removing 1 unit of pTFPI. From Figure 5 it can be seen that the heparin dose-response curves are not parallel in the absence or presence of anti-TFPI IgG. This indicates that a synergism also exists between pTFPI and LMW heparin.

Addition of anti-TFPI IgG to the concentrations of LMW heparin used for the measurements in the PT assay (Figure 2) did not affect the coagulation time

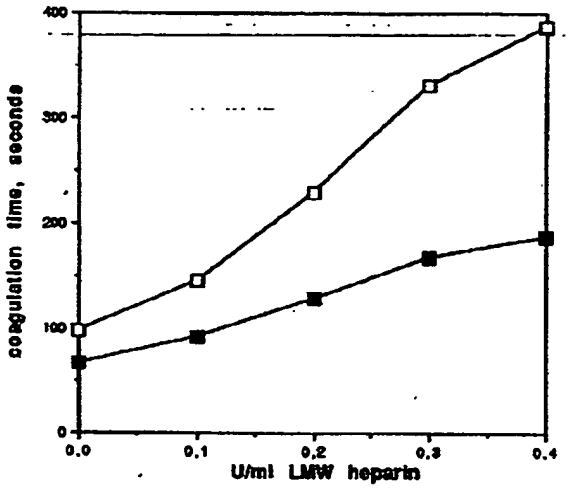

Figure 5. Effect of anti-TFPI IgG on the coagulation time of normal plasma in a dTF assay at different concentrations of LMW heparin. ■, with anti-TFPI IgG; □, without anti-TFPI IgG.

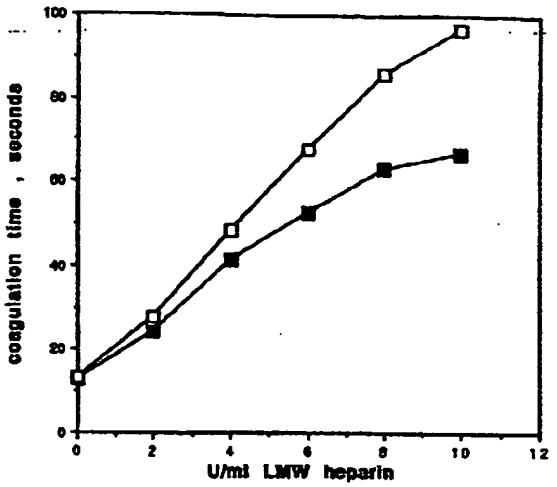

Figure 6. Effect of anti-TFPI IgG on the coagulation time of normal plasma in a PT assay at different concentrations of LMW heparin. ■, with anti-TFPI IgG; □, without anti-TFPI IgG.

significantly. However, if more heparin was added, prolonging the PT of normal human plasma to approximately 100 s, an effect of anti-TFPI IgG was seen in this assay. Figure 6 shows that anti-TFPI IgG in this case shortened the coagulation time by approximately 35 s. The same shortening by anti-TFPI IgG was obtained in the dTF assay where the coagulation time of normal human plasma without added heparin was 100 s (Figure 5).

Discussion

The direct effect of TFPI in coagulation assays was not demonstrated until recently when it was shown that the clotting time of normal plasma, as measured in a dilute tissue factor coagulation assay, could be prolonged by addition of large amounts of rTFPI.[15] It has previously been shown that heparin injection results in increased plasma concentrations of TFPI[9,11] and after neutralizing heparin by addition of polybrene, it was demonstrated that the released TFPI has an anticoagulant activity.[10] It has also been shown that TFPI has a higher affinity for FXa in the presence of heparin.[11] In this study we demonstrate that a synergistic effect on coagulation is obtained when both rTFPI and LMW heparin is added to plasma.

Both in the APTT assay (intrinsic activation), in the PT assay (extrinsic activation) and in a modified PT assay with dilute tissue thromboplastin, the effect of adding rTFPI was potentiated by the addition of LMW heparin (Figures 1, 2 and 3). The synergistic effect of adding both rTFPI and heparin was particularly obvious in the PT assay. Clotting times obtained in coagulation assays may be depicted in many types of plots with linear or logarithmic axis.[16] In both the APTT and dTF assays we used lin–lin plots, while in the PT assay we also depicted the data in a lin–log plot since the latter gave straight lines. However, in all three clotting assays the slopes of the rTFPI dose–response curves were increased when increasing concentrations of heparin was added in both types of plot.

The reported experiments were performed using LMW heparin. However, an experiment with standard heparin in the PT assay (data not shown) indicated that standard heparin also has synergism with TFPI.

A synergistic effect has also been reported between heparin and another Kunitz type inhibitor, aprotinin.[17] Aprotinin inhibits intrinsic coagulation activation by inhibiting kallikrein, and therefore the synergistic effect is seen in the APTT assay.

We used rTFPI from BHK cells for these studies but TFPI originating from different cell lines may show differences in anticoagulant activity.[18] Furthermore, TFPI from human plasma has a 15-fold higher anticoagulant activity than rTFPI produced in BHK cells when measured in a dTF assay.[14,19] Since TFPI has higher anticoagulant activity than rTFPI, we wanted to investigate to what extent heparin affected the anticoagulant activity of pTFPI. For these studies we purified TFPI from plasma using affinity chromatography. However, after this one step purification the anticoagulant activity of pTFPI was unstable, and further purification to obtain stable pTFPI will be necessary before investigation of the effect f simul-

not monitor the anticoagulant activity. The reason for the instability of pTFPI is not known at present, nor is it known if there is any relationship between this instability and the differences in anticoagulant activity of TFPI obtained from different cell lines.[18] The instability of pTFPI was only observed in coagulation assays and not in the two-stage chromogenic assay. Thus the pTFPI was still able to inhibit FXa and FVIIa although the anticoagulant activity was significantly reduced. This may indicate that the high anticoagulant activity of pTFPI was caused by faster reaction kinetics or inhibition of other factors than FXa and FVIIa. There may be several reasons for the observed instability of partly purified pTFPI; changes in composition of the lipoprotein component, proteolytic degradation, conformational changes or other explanations. These aspects are presently under investigation in our laboratory.

To obtain an impression of the importance of heparin for the anticoagulant activity of pTFPI, we added heparin to normal human plasma and investigated the effect of inhibiting pTFPI with the use of antibodies towards TFPI. For this experiment we chose to use the dTF assay since it is very sensitive to TFPI. We observed that the dose–response curve for heparin was steeper for the normal human plasma than for the plasma with added anti-TFPI IgG, showing that a synergism also exist between pTFPI and heparin in coagulation.

There may be two reasons for the synergism observed in the different assays. One reason may simply be that heparin increases the affinity for FXa as shown by Broze *et al.*[11] Another may be that heparin, by forming complexes with antithrombin III, which inhibits FXa and thereby thrombin generation as well as the important feedback activation of FV and FVIII, prolongs the coagulation time and thereby gives TFPI more time to form complexes with FXa and thus to become an inhibitor of the FVIIa–TF complex.

If sufficient heparin is added to plasma in the PT assay to prolong the coagulation time to near 100 s, then anti-TFPI IgG shortened the coagulation time by approximately 35 s. This shortening was similar to that obtained in the dTF assay where the coagulation time without heparin was prolonged to around 100 s by using very small amounts of tissue thromboplastin.

rTFPI has antithrombotic properties both in an endotoxin model[19] and in a model of venous thrombosis.[20] Compared to the plasma concentration of TFPI, relatively high concentrations of rTFPI were used to obtain the antithrombotic effect. Our data is particularly obvious when the coagulation time is prolonged as also exemplified by dTF clotting of haemophilia plasma.[14] In normal circulating blood the clotting time is infinite and our data suggest that in this situation TFPI is an important factor for preventing clotting.

Considering also the increased TFPI concentration after heparin injection, we put forward the hypothesis that TFPI may contribute considerably to the antithrombotic effect of heparin.

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